Molecular and Population Genetic Aspects of Mitochondrial DNA Variability in the Diamondback Terrapin, *Malaclemys terrapin*

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Diamondback terrapins (*Malaclemys terrapin*) occupy brackish waters along North America's Atlantic and Gulf coasts. Despite nearly continuous distribution, terrapin populations exhibit extensive geographic variation, with seven subspecies recognized. To assess population-genetic structure in *Malaclemys*, we used 18 restriction enzymes to assay mitochondrial DNA (mtDNA) genotypes in 53 terrapins collected from Massachusetts to western Louisiana. MtDNA size polymorphism and heteroplasmy were observed, attributable to variation in copy number of a 75-bp tandem repeat. In terms of restriction sites, mtDNA genotypic diversity ($G = 0.582$) and divergence levels ($p < 0.004$) were exceptionally low. Only one restriction site polymorphism appeared geographically informative, clearly distinguishing populations north versus south of Florida's Cape Canaveral region. Nonetheless, the probable zoogeographic significance of this single site change is underscored by its (1) perfect concordance with the distribution of a key morphological character and (2) striking agreement with phylogeographic patterns observed for mtDNA profiles of several other coastal marine species. The possible isolation of Atlantic and Gulf terrapin populations during late-Pleistocene glacial maxima conceivably accounts for the observed patterns of mtDNA (and morphological) variation.

During the early 1900s, recognition of geographic races pervaded systematics, and much research was directed toward the identification and taxonomic description of intraspecific variation. This preoccupation, in its extreme form, resulted in up to 150 trinomial assignments within a species (Goldman 1935). Biologists today must contend with the nomenclatural legacies left by the zealous taxonomic activities of this period. In many cases, geographic races were described on the basis of subtle (or plastic) morphological distinctions such that their status as valid evolutionary units must be questioned. In other cases, original subspecific designations appear legitimate upon taxonomic reappraisal, as distinctive character complexes (genetic and/or morphological) with long-term adaptive or historical bases are uncovered.

In this article we examine patterns of mitochondrial DNA (mtDNA) variation among populations assignable to the seven subspecies of the diamondback terrapin, *Malaclemys terrapin* (Figure 1). *Malaclemys* exhibits extensive variation in external appearance, both in pigmentation and shell shape patterns (Ernst and Barbour 1989; Wood 1977). Clear geographic variation also is evident, involving pronounced characters that consistently differentiate various subspecies (Ernst and Barbour 1989). Indeed, certain subspecies are so distinct that they were treated as separate species (Hay 1904).

One peculiar feature about geographic variation in *Malaclemys* is the geographic setting in which this variation persists and possibly arose. Diamondback terrapins are confined to a narrow strip of brackish (estuarine) coastal waters that forms a rather continuous habitat from Cape Cod to western Texas (Ernst and Barbour 1989). Such a connected distribution pattern and its potential for genetic exchange seem at odds with the morphological differentiation observed in this species.

The purposes of this report are to (1) examine the levels and possible geographic components of mtDNA variation among recognized subspecies of *Malaclemys* and (2) determine whether mtDNA divergence in the terrapin exhibits congruent phylogeographic patterns with those of other coastal marine species previously surveyed (Avise 1992). Additionally, molecular features of mtDNA, including size polymorphism and heteroplasmy, are described.

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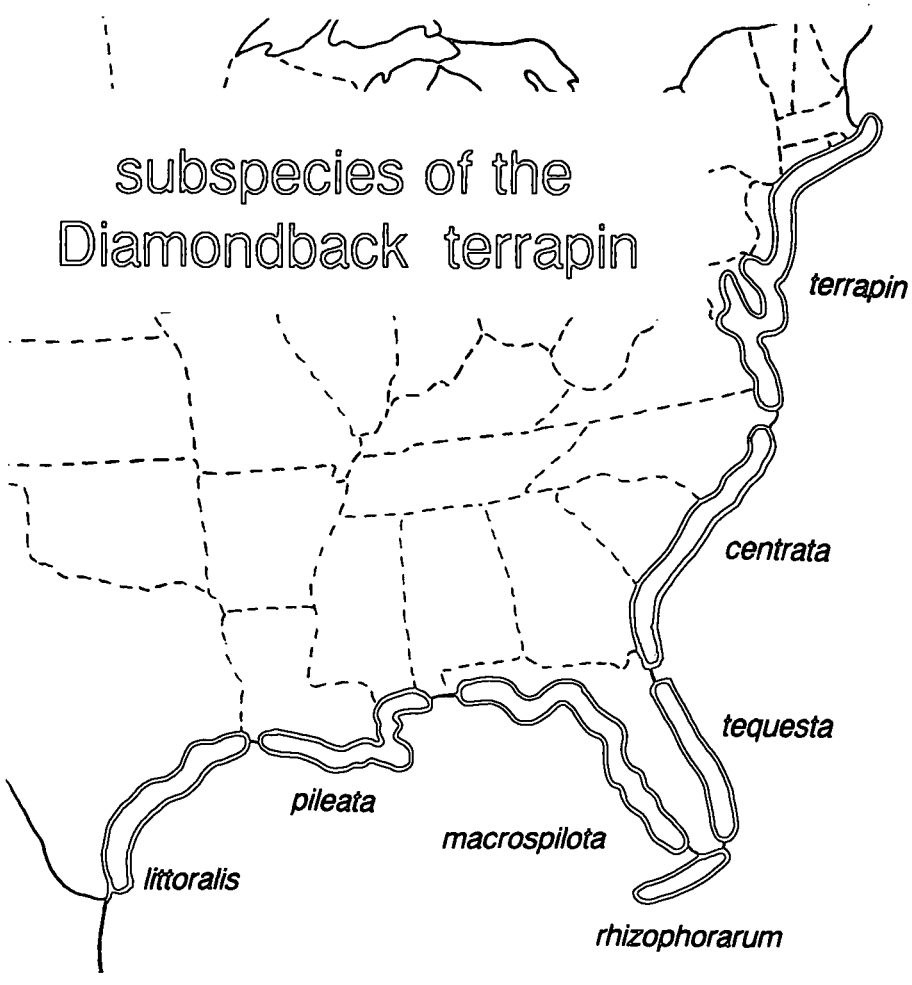

Figure 1. Geographic distribution of the seven currently recognized subspecies of *Malaclemys terrapin*, based on morphological characters.

Materials and Methods

We collected 53 diamondback terrapins from the following locales from Massachusetts to Louisiana: Barnstable Co., Massachusetts (N = 2); Gloucester Co., Virginia (N = 5); Charleston Co., South Carolina (locale 1, N = 8; locale 2, N = 3); Chatham Co., Georgia (N = 5); Camden Co., Georgia (N = 2); Brevard Co., Florida (N = 8); Monroe Co., Florida (N = 4); Hillsborough Co., Florida (N = 9); Levy Co., Florida (N = 1); Franklin Co., Florida (N = 1); Harrison Co., Mississippi (N = 3); Iberia Parish, Louisiana (N = 1); and Vermillion Parish, Louisiana (N = 1).

We used the CsCl-gradient approach described in Lansman et al. (1981) to isolate mtDNA in closed-circular form from fresh heart or liver tissue. Purified mtDNAs were digested individually with 18 restriction enzymes that revealed two or more recognition sites in the molecule. In addition, we used *Bam*HI, *Eco*RI, and *Xba*I, but they are not considered further since each produced only zero or one mtDNA restriction fragment in our assays. We conducted all restriction digests overnight under conditions recommended by the enzyme suppliers. The mtDNA fragments were end labeled with ^{35}S-radionucleotides, separated through 1%–1.8% agarose gels, and revealed by autoradiography (Brown 1980; Lansman et al. 1981; Maniatis et al. 1982). We compared fragment sizes to those in a 1-kb molecular weight standard (Bethesda Research Labs). Restriction sites were mapped by analyses of "double digests" from various pairs of endonucleases employed jointly.

To test for a possible duplicated region in the mtDNA molecule (see Results), we cloned a 4.2-kb *Pst*I fragment of *Malaclemys* mtDNA into a modified pUC18CM plasmid cloning vector (which exhibits chloramphenicol resistance, and was kindly provided by K. J. Buckley (Buckley 1985; Buckley and Hayashi 1986). This probe was subsequently used in Southern blot hybridizations (Maniatis et al. 1982) against the total *Malaclemys* mtDNA digested with particular endonucleases. Hybridizations were conducted under low stringency conditions (one filter wash at room temperature for 30 min).

We calculated estimates of nucleotide sequence divergence (p) by the restriction "site" approach of Nei and Li (1979). Mean sequence divergence between individuals (nucleotide diversity as in Nei 1987) was calculated separately for Atlantic and Gulf collection locales and converted to estimates of female evolutionary effective population size ($N_{f(e)}$), following Avise et al. (1988). The latter estimates assumed a conventional mtDNA rate calibration (2% sequence divergence between lineages per million years, per Wilson et al. 1985), and a 5-year generation length for terrapins. Values of genotypic diversity (G) were calculated as

$$G = n(1 - \Sigma f_i^2)/(n - 1)$$

where f_i is the frequency of the ith mtDNA genotype among the n specimens assayed (Nei and Tajima 1981). Genotypic diversity gives the probability that two randomly drawn individuals from the sample exhibit the same mtDNA genotype.

Results

Molecular Features of Terrapin mtDNA

The most common mtDNA genotype in *Malaclemys* consisted of a total of 74 observed restriction fragments, produced by the following enzymes: *Ava*I (3 fragments); *Ava*II (8); *Bcl*I (4); *Bgl*I (2); *Bgl*II (2); *Bst*EII (3); *Cla*I (2); *Hin*cII (2); *Hin*dIII (3); *Kpn*I (2); *Msp*I (11); *Nde*I (5); *Pst*I (2); *Puv*II (4); *Sac*I (3); *Spe*I (7); *Sst*II (2); and *Stu*I (9). As judged by numerous single (and double) digests, the average mtDNA size in *Malaclemys* is about 16.8 kb, which is quite typical for most metazoan animals (Brown 1983). However, there was some variation about this length, both within and among individuals.

The mtDNA size differences and heteroplasmy were most evident in gel profiles where the variable-length region happened to occur in the small, better separated fragments. For example, four size classes were observed in *Spe*I digests in the 1.2–1.5-kb region (Figure 2). The mtDNA bands were discrete and evenly spaced, indicating a tandem repeat unit of about 75 base pairs (bp). The size variation was also especially clear in digestion profiles produced by *Ava*II, *Bcl*I, and *Stu*I.

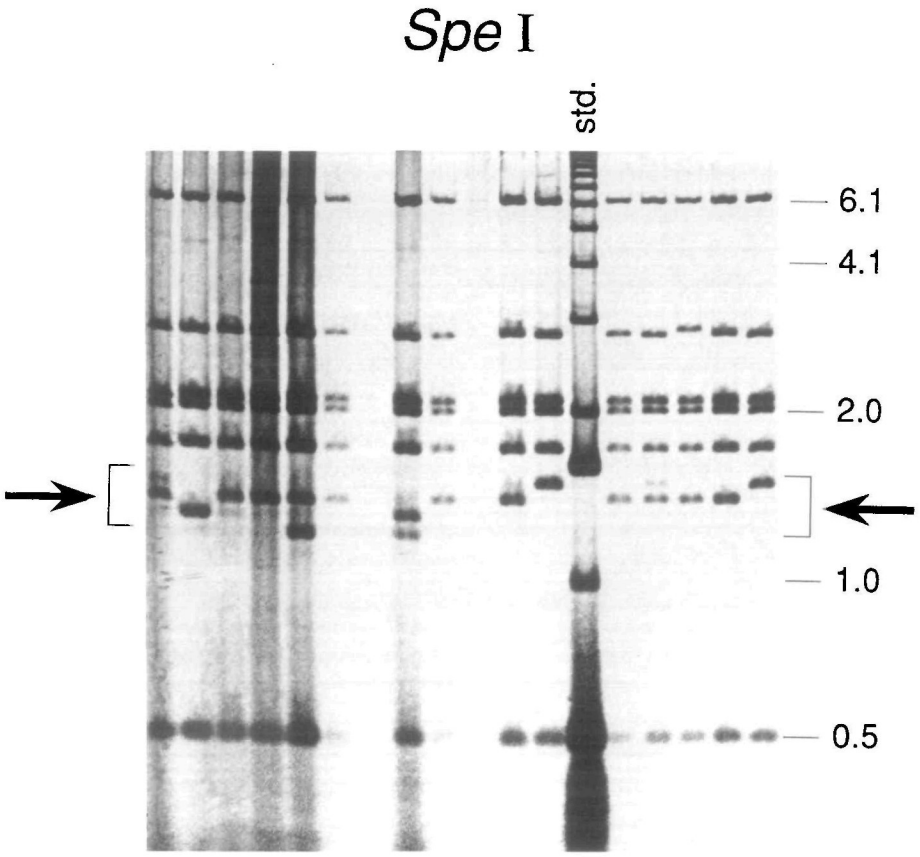

Figure 2. *Spe*I digests of *Malaclemys* mtDNA. The region in brackets exhibits the size polymorphism and heteroplasmy. Numbers refer to selected fragment lengths (kb) of the molecular size standard in lane 6 from the right.

A concordance across individuals in the digestion profiles produced by separate endonucleases confirmed that these differences were due to localized mtDNA length differences (Figure 3).

At least 17 individuals (32%) were unambiguously heteroplasmic for mtDNA size variants (Table 1). However, the relative proportions of different size classes within heteroplasmic individuals (as judged by relative band intensities) appeared to vary considerably (Figure 2), such that additional heteroplasmic individuals with low proportions of one or another size class likely were present but undetected. Most heteroplasmic individuals exhibited two mtDNA size classes, but three such classes were visible in at least one specimen. The smaller size classes were significantly more frequent in the Gulf than in the Atlantic

Table 1. Distribution of the mtDNA size polymorphism and heteroplasmy involving the tandem repeat unit of 75 bp[a]

Atlantic locale	mtDNA genotypes[b]	Gulf locale[c]	mtDNA genotype[b]
Massachusetts	2; 2	Florida (Brevard)	2; 2; 2/4; 2/3/4
Virginia	2; 2; 2; 1/2/3; 1/2	Florida (Monroe)	3; 3; 3/4; 3/4
South Carolina, locale 1	1; 1; 2; 2; 2; 1/2; 3; 3	Florida (Hillsborough)	1/2; 2/3; 2/3; 2/3; 2/3; 2; 3; 3; 3
South Carolina locale 2	1; 1/2; 1/2	Florida (Levy, Franklin)	2; 2
Georgia	2; 2; 2; 2; 2	Mississippi	1/2; 1/2; 2
		Louisiana	2; 2

[a] Only 47 individuals were scored for mtDNA size class.

[b] Semicolons separate genotypes of different individuals. Numbers indicate size classes, with "1" the largest and "4" the smallest size class. Heteroplasmic specimens show two or three size classes (separated by slashes), with the predominant size class underlined when they differed clearly in abundance.

[c] "Gulf" locales listed here include the Brevard and Monroe, Florida, populations which exhibit the *Bst*EII-C pattern (see text). The incidence of smaller size classes (3 and 4) is significantly greater in the Gulf than in the Atlantic collections [$G = 24.2$, df = 1, $P < .001$ (Sokal and Rohlf 1969)].

(Table 1). The size-variable region maps close to, and most likely within, the D loop (or "control region," Brown 1985) of the mtDNA molecule. In recent years, similar examples of localized mtDNA size variation and heteroplasmy, usually in the control region, have been reported for a number of vertebrate and invertebrate species (Avise and Zink 1988; Bermingham et al. 1986; Biju-Duval et al. 1991; Harrison 1989; Moritz et al. 1987).

Oddly, four enzymes—*Ava*I, *Bgl*II, *Cla*I, and *Hin*dIII—produced nearly identical gel profiles involving two mtDNA fragments of approximate sizes 8.8 and 8.0 kb. (*Ava*I also exhibited a small fragment about 0.3 kb in size.) Double digests involving all six possible pairs of these enzymes produced a "cascading" gel pattern (Figure 4). To characterize further the molecular basis of these features, we mapped *Malaclemys* restriction sites relative to one another by a series of double digests involving these and other enzymes. The *Malaclemys* map was then aligned to the known gene maps of other vertebrates using two highly conserved *Sst*II sites (one in each rRNA gene), which appear to be present nearly universally (Wallis 1987). Additional alignment of the *Malaclemys* mtDNA against that of *Xenopus* was facilitated by two apparently conserved *Cla*I sites (Wallis 1987).

The *Ava*I, *Bgl*II, *Hin*dIII, and *Cla*I sites group into two distinct "modules" (each hereafter designated A-B-H-C) that occur on nearly opposite sides of the mtDNA molecule (Figure 5). The spacing and order of these four sites appear essentially identical in the two modules, accounting for the cascading gel profile in Figure 4. To test whether the A-B-H-C region could represent a large-scale duplication, we cloned a 4.1-kb region (the smaller of two *Pst*I fragments) surrounding the A-B-H-C module in the cytochrome c oxidase-N3 area. This clone, used as a probe, was hybridized against total *Malaclemys* mtDNA digested with *Pst*I, *Bcl*I, and *Kpn*I. The resulting autoradiograph revealed only the expected bands in the probe region; we observed no detectable traces of hybridization with fragments encompassing the second A-B-H-C module (Figure 6). Thus, we tentatively conclude that the A-B-H-C alignments represent only a fortuitous, parallel arrangement of restriction sites.

Population Genetic Features of Terrapin mtDNA

The 73–75 restriction sites scored per individual represent about 400 bp of infor-

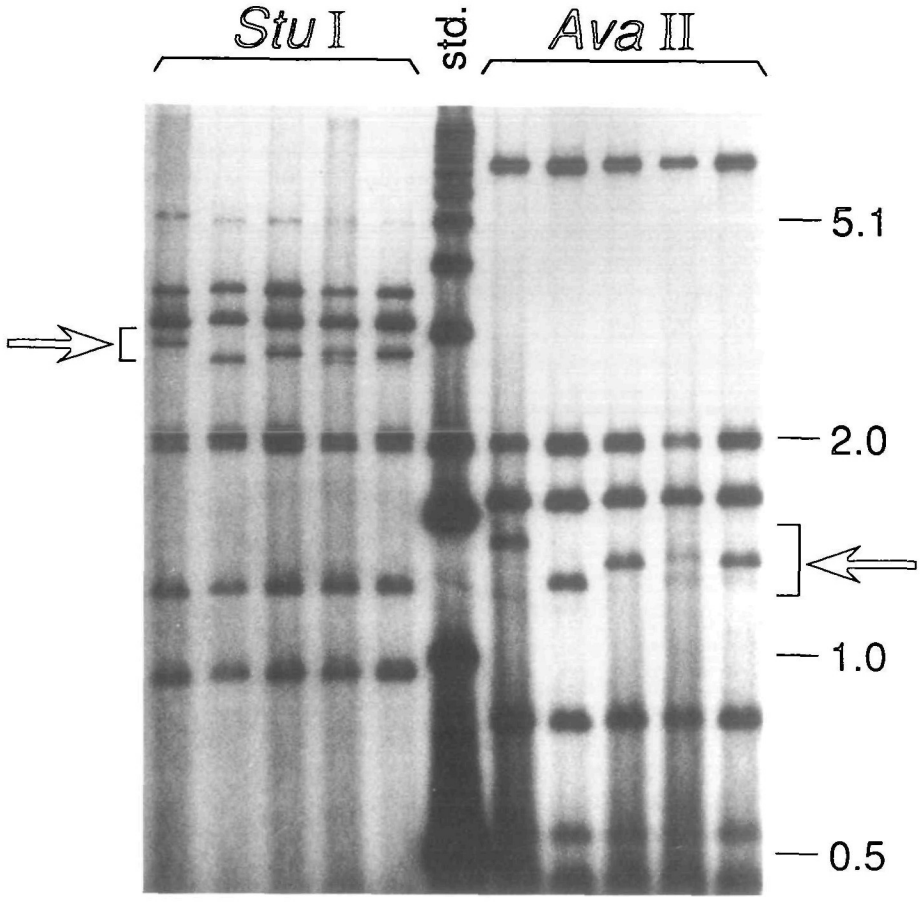

Figure 3. *Stu*I and *Ava*II digests of *Malaclemys* mtDNA. Five individuals are shown, arranged in the same order from left to right in digests for the two enzymes. The regions indicated by arrows exhibit the concordant band shifts resulting from the size polymorphism and heteroplasmy. Numbers refer to selected fragment lengths (kb) of the molecular size standard in the middle lane.

mation in recognition sequence assayed, or 2.4% of the mitochondrial genome. Restriction site variation was limited, as evidenced by the appearance of only six different mtDNA genotypes among 53 assayed specimens. With one exception, each mtDNA site variant occurred in a single individual: one turtle from Hillsborough Co., Florida, exhibited the gain of a *Hin*cII restriction site; another specimen from that locale showed an *Ava*I site gain; one specimen from Charleston Co., South Carolina, showed both an *Ava*I site loss and a *Hin*dIII site gain; and one turtle from Franklin Co., Florida, showed a variant *Ava*I pattern explainable by two site changes, one gain and one loss from the common genotype at that locale.

The remaining restriction site variant involved *Bst*EII, where two common patterns differed by a single site change: the genotype *Bst*EII-"*C*" exhibited three fragments of length 6.8, 6.2, and 3.8 kb, whereas *Bst*EII-"*D*" had 13.0- and 3.8-kb fragments. All 25 terrapins from northern Florida to Massachusetts possessed "D" genotypes; conversely, "C" genotypes were restricted to the 28 terrapins from Cape Canaveral to western Louisiana (Figure 7).

Estimates of nucleotide sequence divergence were uniformly low, the maximum value being only $p = 0.004$. Most *Malaclemys* individuals were identical at all restriction sites, or else differed only by the *Bst*EII site change. Estimates of $N_{f(e)}$ for Atlantic and Gulf collections, derived from nucleotide diversity values and assuming a conventional clock, were 1,000 and 3,000 females, respectively. If a slower clock is assumed (see Discussion), these values should be adjusted upward by a corresponding factor. Overall genotypic diversity was 0.582, which is among the lower values reported for a vertebrate species (Avise et al. 1989), and virtually all of the diversity was attributable to the two *Bst*EII genotypes that were nearly equally frequent in our samples.

Discussion

The diamondback terrapin exhibits an unusually low level of mtDNA variability in comparison to most other vertebrates (Avise et al. 1987, 1989; Moritz et al. 1987). Of the limited site polymorphisms detected, only the *Bst*EII variant was geographically informative, with a distinct "break" between genotypes *C* and *D* near Cape Canaveral, Florida. Nonetheless, the possible evolutionary significance of this single mtDNA character is underscored by (1) its distribution among the terrapin subspecies and (2) dramatic phylogeographic similarities in terrapin mtDNA with significant population subdivisions observed in other coastal marine animals.

Genetic versus Morphological Variation in *Malaclemys*

The limited mtDNA differentiation in *Malaclemys* initially appears inconsistent with the magnitude and pattern of morphological differentiation in this species. However, the geographic pattern observed for the *Bst*EII polymorphism is perfectly concordant with a key morphological character distinguishing mid-Atlantic *Malaclemys* populations from those in central Florida and the Gulf coast.

The Florida East Coast Terrapin (*M. t. tequesta*), whose range extends from the Cape Canaveral area to the Keys, possesses a series of tubercles on the medial keel of the carapace (dorsal shell) (Schwartz 1955). This distinctive feature, absent in subspecies farther north (*M. t. terrapin, M. t. centrata*), becomes increasingly pronounced in the Keys and Gulf coast races (east to west: *M. t. rhizophorarum, macrospilota, pileata,* and *littoralis*) (Figure 1; Ernst and Barbour 1989). Similarly, the *Bst*EII-"*C*" genotype characterizes the two mid-Atlantic subspecies, whereas *Bst*EII-"*D*" first appears in the northern range of *M. t. tequesta* and is apparently fixed in those subspecies bearing tuberculate keels. If we presume that the tuberculate condition of the keel has a strong genetic basis, such geographic concordance between the genealogies of supposedly independent character states provides support for significant historical population partitioning (Avise and Ball 1990).

Genetic variation revealed in our mtDNA assay did not reflect the fine-scale geographic patterns apparent for morphological variation in *Malaclemys*. Aside from the dorsal keel condition, most morphological traits distinguishing terrapin subspecies are based on shell and skin pigmentation patterns. The presence of morphological differences in the absence of mtDNA differences is open to alternative interpretations. First, perhaps some of the morphological variation is environ-

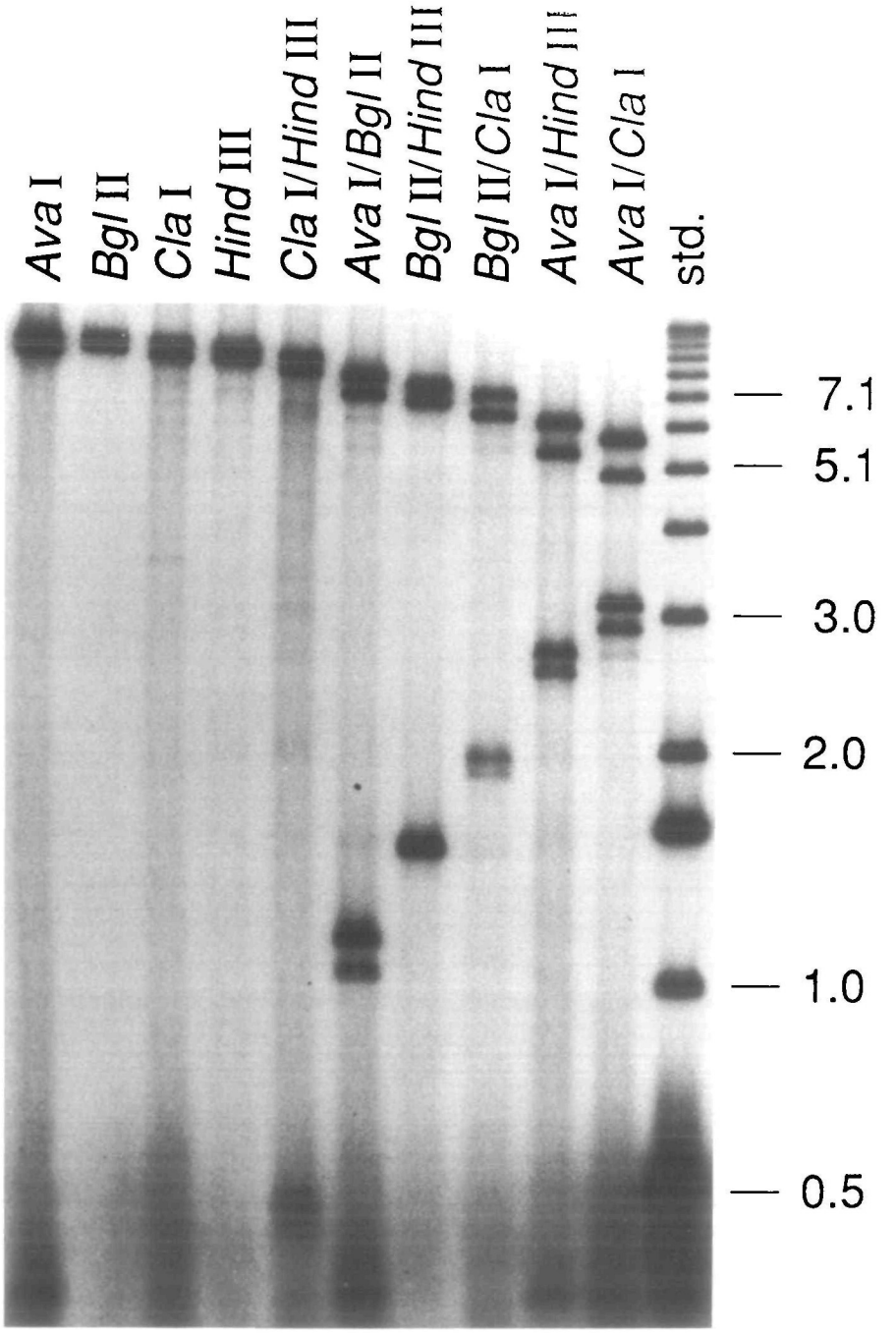

Figure 4. Single and double digests of *Malaclemys* mtDNA. Numbers refer to selected fragment lengths (kb) of the molecular size standard in the lane on the far right.

mentally rather than genetically based. Second, given the enormous amount of color polymorphism within certain *Malaclemys* populations (Wood 1977; T. Lamb, personal observation), there is at least the potential for rapid, localized changes in pigment patterns. Either strong selection or genetic drift influencing genetically based color morphs could operate over time scales too shallow for the accumulation of de novo mtDNA mutations.

Third, mtDNA evolution in *Malaclemys* may be slower than is conventionally assumed for other vertebrates. Avise et al. (1992) provide evidence for about an eightfold deceleration in mtDNA microevolutionary rate for several marine, freshwater, and terrestrial turtles. Thus, our mtDNA assay simply may have failed to resolve genetic differences that truly exist among the terrapin subspecies. Unfortunately, a comparable allozymic survey of geographic variation has not been conducted for this species.

Comparisons to Other Coastal Marine Species

Perhaps the most intriguing aspect of the geographic structure observed for *Malaclemys* mtDNA is its striking similarity to patterns of mtDNA variation in a variety of other coastal marine forms (review in Avise 1992). MtDNA phylogeographic profiles for the American oyster (*Crassostrea virginica*) (Reeb and Avise 1990) and horseshoe crab (*Limulus polyphemus*) (Saunders et al. 1986) essentially mirror that of *Malaclemys*: diagnostic mtDNA clades characteristic of mid-Atlantic versus Gulf mtDNA assemblages abut Florida's east coast near Cape Canaveral. Major lineage partitioning between Atlantic and Gulf populations is also evident in mtDNA surveys for black sea bass (*Centropristis striata*) (Avise 1992) and seaside sparrow (*Ammodramus maritimus*) (Avise and Nelson 1989).

Avise et al. (1987) proposed that concordant patterns detected among the intraspecific phylogenies of ecologically similar species may reveal historical features that figure prominently in regional biogeography. Geographic concordance among mtDNA phylogenies of the above taxa point to peninsular Florida (in general) and the Cape Canaveral area (in particular) as regions of substantive zoogeographic influence. The Cape Canaveral region currently functions as an ecological transition zone, demarcating northern and southern range boundaries for many tropical and temperate marine species. Moreover, historical expansion and contraction of the Florida peninsula, in response to Pliocene-Pleistocene sea level fluxes, likely provided barriers (as well as corridors) to dispersal and gene flow for southeastern marine fauna (Avise 1992; Bert 1986).

One plausible vicariant explanation for mtDNA differentiation in the American oyster (and other species) involves Pleistocene glacial maxima (Reeb and Avise 1990). During these periods, sea level in the Gulf dropped some 150 m, exposing extensive portions of the West Florida Shelf as well as northern portions of the Yucatan Peninsula (Poag 1973). This land mass expansion, coupled with increased aridity in the southeast (Watts 1980) and hypersaline conditions at the mouth of the Gulf (Poag 1981), likely isolated the Gulf's estuarine ecosystems from those along the Atlantic. Such a setting may have split ancestral terrapin populations as well, accounting for the morphological and mtDNA distinctions between Gulf and mid-Atlantic subspecies. It is possible that *M.*

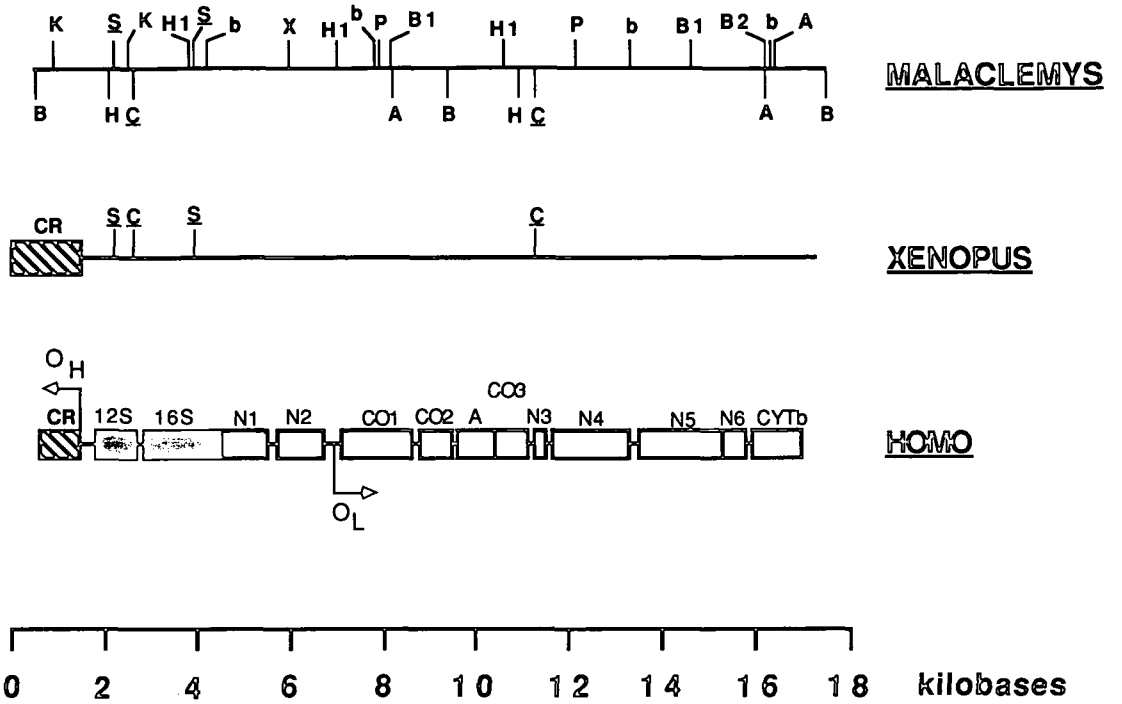

Figure 5. MtDNA restriction site map for *Malaclemys terrapin,* aligned against maps for the clawed frog [*Xenopus laevis* (Roe et al. 1985)] and humans (Anderson et al. 1981). Genes in the *Homo* map are designated as follows: N1-N6, NADH dehydrogenases; CO1-CO3, cytochrome c oxidases; CYTb cytochrome b; A, ATP synthase; 12S and 16S, ribosomal RNAs; CR, control region. O_H and O_L refer to the origins of heavy and light strand replication. In the chicken and some other birds, the N6 gene (and adjacent $tRNA^{glu}$) occur next to the CR rather than between CYTb and N5 (Desjardins and Morais 1990, 1991). Restriction sites in the *Malaclemys* and *Xenopus* maps are designated as follows: A, *Ava*I; B1, *Bgl*I; B, *Bgl*II; b, *Bcl*I; B2, *Bam*HI; C, *Cla*I; H, *Hin*dIII; H1, *Hin*cII; K, *Kpn*I; P, *Pst*I; S, *Sst*II; and X, *Xba*I.

t. tequesta, which possesses both morphological and genetic characteristics of Gulf terrapins, represents recent dispersal around south Florida from Gulf *Malaclemys* stock.

There is little question that the pattern of geographic structure of mtDNA variation in *Malaclemys* is shared with a number of co-distributed species. Yet the magnitude of mtDNA divergence between Atlantic and Gulf populations of *Malaclemys* is considerably lower than that of other surveyed species. Assume for the sake of argument that the conventional mtDNA "clock" (about 2% sequence divergence between mammalian and avian lineages per million years—Brown et al. 1979; Wilson et al. 1985) applies to other vertebrates as well. Then, lineage separation in *Malaclemys* dates to less than 50,000 years ago, whereas separations for the other coastal marine taxa range from 350,000 to 1,100,000 years before present (Table 2).

Two classes of explanation might account for such discrepancies in estimates of absolute divergence time. First, about 10 separate glacial advances have been documented for the Pleistocene epoch, each with similar climatic and geographic impacts (Hoyt and Hails 1967). Assuming recurrent estuarine isolation, it is possible that lineage separations for various coastal marine taxa were established during different glacial regimes. Thus, mtDNA differentiation in *Malaclemys* may have been shaped by a later glacial episode than were the other taxa. The wide range of divergence estimates for the species surveyed is consistent with this explanation (Table 2).

Alternatively, the discrepancies in magnitude of mtDNA divergence across the Atlantic/Gulf boundary may involve taxonomic differences in rate of mtDNA evolution. For example, essentially all turtle species surveyed to date exhibit exceptionally low levels of intraspecific mtDNA polymorphism and differentiation (Avise et al. 1992; Bowen and Avise 1990; Bowen et al. 1989; Lamb et al. 1989). Elsewhere we summarize evidence and formalize an argument for a severalfold deceleration in mtDNA evolutionary rate in the turtles (order Testudines) (Avise et al. 1992).

In conclusion, although the mtDNA phylogeographic pattern for *Malaclemys* exhibits remarkable aspects of concordance with those of several other coastal animal species, notable differences also exist. Through range-wide surveys of co-distributed species, we should gain a better appreciation of how the intricacies of ecological and historical influence can variously shape associations between geography, morphology, and genetics.

Table 2. Estimates of mtDNA sequence divergence (p) (corrected for within-region polymorphism)[a] and provisional separation times between Gulf versus mid-Atlantic assemblages of estuarine and coastal marine species

Species	p	Separation times (years)
Diamondback terrapin (*Malaclemys terrapin*)	0.001	50,000
Black sea bass (*Centropristis striata*)	0.007	350,000
Seaside sparrow (*Ammodramus maritimus*)	0.010	500,000
Horseshoe crab (*Limulus polyphemus*)	0.016	800,000
American oyster (*Crassostrea virginica*)	0.022	1,100,000

[a] $p_{corr} = p_{xy} - 0.5(p_x + p_y)$, where p_x and p_y are the mean pairwise genetic distances of mtDNA haplotypes within regions x and y, respectively.

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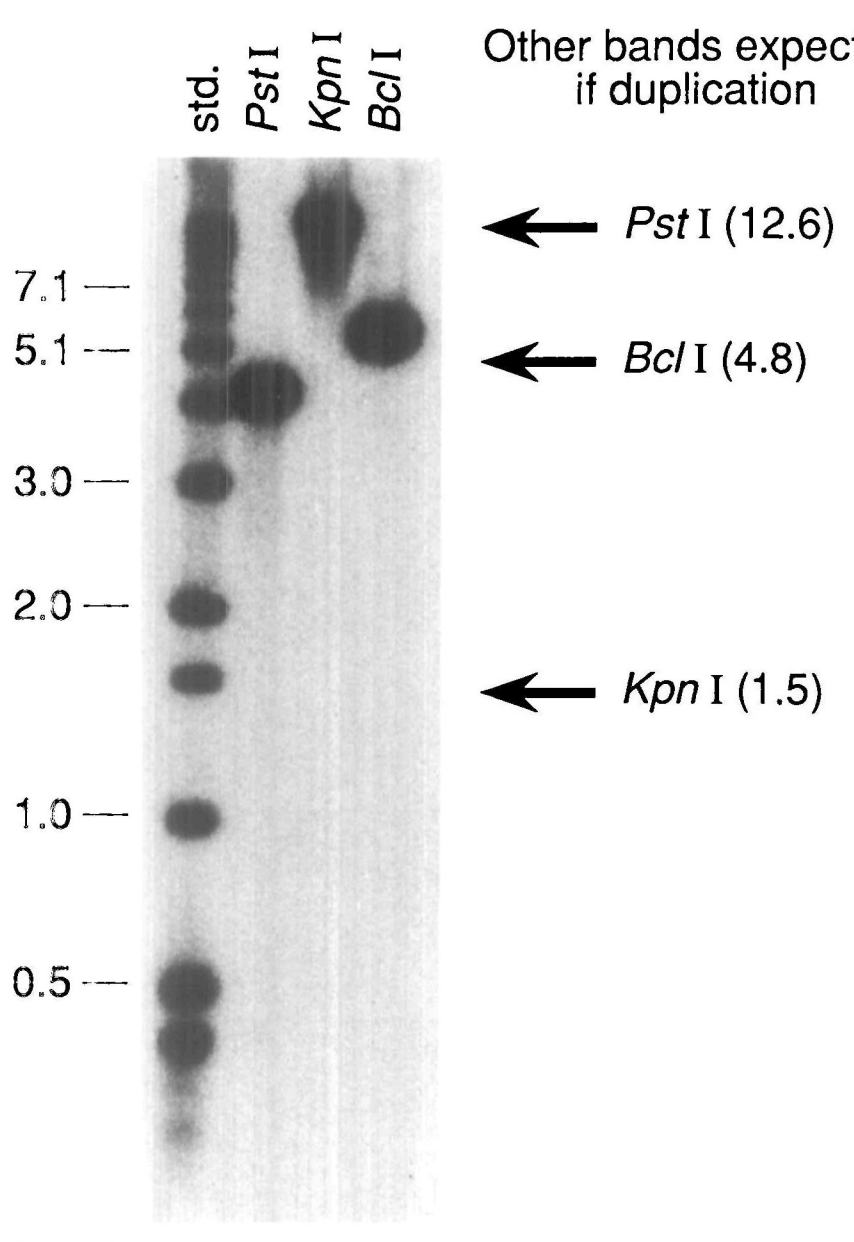

Figure 6. Overexposed Southern blot of *Malaclemys* mtDNA digested with *Pst*I, *Bcl*I, and *Kpn*I, and hybridized against the cloned 4.2-kb *Pst*I fragment (see Figure 5). Arrows indicate where additional bands (and their sizes, in kb) should have appeared for the indicated enzymes had there been the hypothesized region of duplication in the molecule. Numbers on the left refer to selected fragment lengths (kb) in the molecular size standard in the lane on the far left.

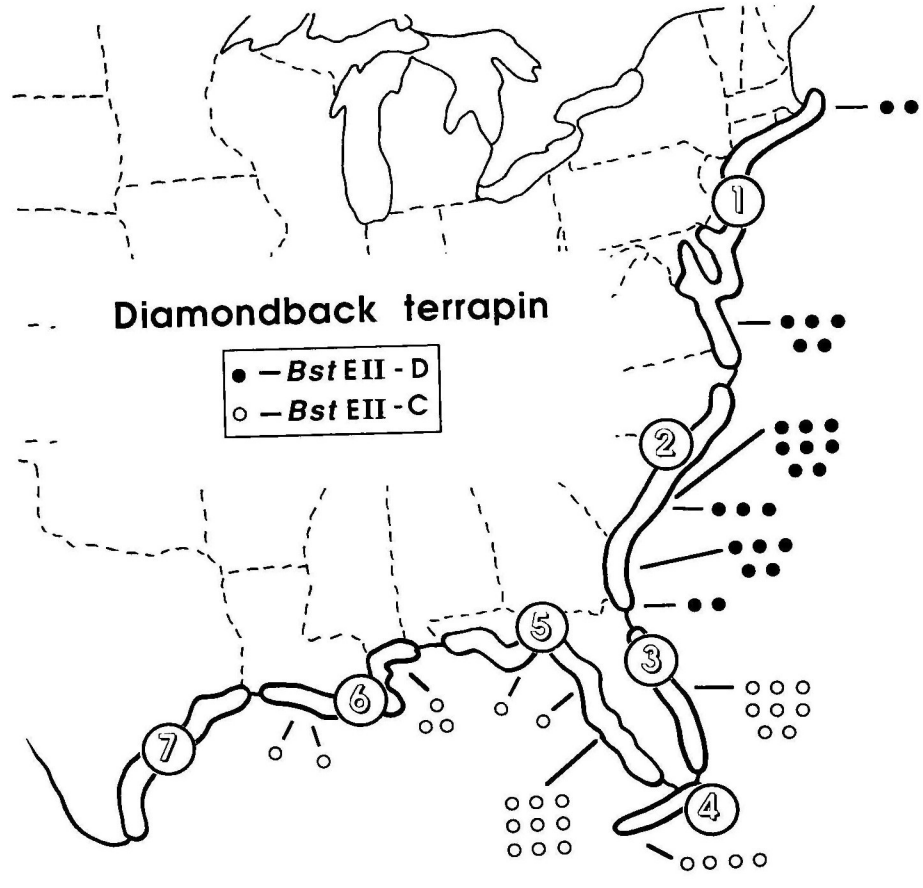

Figure 7. Geographic distributions of the *Bst*EII-C and -D mtDNA genotypes observed in *Malaclemys terrapin*. The subspecies *M. t. tequesta* is designated by number 3.

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